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Low-Level Expression of MicroRNAs *let-7d* and *miR-205* Are Prognostic Markers of Head and Neck Squamous Cell Carcinoma

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Small noncoding microRNAs (miRNAs) have been shown to be abnormally expressed in every tumor type examined. The importance of miRNAs as potential cancer prognostic indicators is underscored by their involvement in the regulation of basic cellular processes such as cell proliferation, differentiation, and apoptosis. In this study, miRNA expression profiles of head and neck squamous cell carcinoma (HNSCC) tumor and adjacent normal tissue were examined by microarray analysis and validated by quantitative TaqMan real-time polymerase chain reaction. Using TaqMan real-time polymerase chain reaction we measured the quantitative associations between a subset of miRNAs identified on microarrays in primary tumors at diagnosis and cancer survival in a cohort of 104 HNSCC patients undergoing treatment with curative intent. The majority of miRNAs exhibiting altered expression in primary human HNSCC tumors (including *miR-1*, *miR-133a*, *miR-205*, and *let-7d*) show lower expression levels relative to normal adjacent tissue. In contrast, *hsa-miR-21* is frequently overexpressed in human HNSCC tumors. Using univariate and multivariable statistical models we show that low levels of *hsa-miR205* are significantly associated with loco-regional recurrence independent of disease severity at diagnosis and treatment. In addition, combined low levels of *hsa-miR-205* and *hsa-let-7d* expression in HNSCC tumors are significantly associated with poor head and neck cancer survival Our results show that miRNA expression levels can be used as prognostic markers of head and neck cancer. *(Am J Pathol 2009, 174:736–745; DOI: 10.2353/ajpath.2009.080731)*

MicroRNAs (miRNAs) are a group of noncoding 22 nucleotide RNA molecules that posttranscriptionally regulate the expression of target mRNA.[1] These newly discovered small RNAs regulate processes as fundamental as cellular proliferation, differentiation, and apoptosis, and a subset of miRNAs have been identified as potential diagnostic and prognostic markers in cancer.[2–4]

Among these, recent studies have shown that overexpression of *miR-21* in lung cancer tumors may act as a classic oncogene, and down-regulation of *let-7* miRNA family of genes, which target the *Ras* oncogene, may be correlated with poor survival and relapse in non-small cell lung cancer.[5,6] Aberrant regulation of miRNAs has also revealed roles for specific genes as either tumor suppressors, when deleted or repressed, or as oncogenes when amplified or otherwise overexpressed.[2,7,8] However, miRNA expression is highly tissue-specific; separate and distinct profiles have been described for every cancer type.[9] To date there have been only two studies investigating miRNAs in head and neck squamous cell carcinoma (HNSCC) immortal cell lines, neither of which assessed the role of these noncoding RNA molecules in human tissue or attempted to correlate miRNA expression with HNSCC prognosis.[9,10]

HNSCC is the fifth most common malignancy in men worldwide and includes tumors of the oral cavity, oropharynx, and larynx. Survival rates for HNSCC have remained unchanged throughout the last 3 decades, and half of all cases die within 5 years of diagnosis.[11] Efforts to identify prognostic biomarkers for HNSCC, including

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p53, *EGFR*, *Bcl-2*, *MMPs*, cyclins, and other markers, have proved to be primarily unsuccessful to date.[12–14] As with mRNA expression, high-throughput genomic technologies can be used to shed new light on alterations in gene expression that are associated with HNSCC carcinogenesis.[15–17] This is the first report to comprehensively identify and measure differentially transcribed miRNAs in HNSCC and assesses their role in cancer prognosis in humans.

Materials and Methods

Collection of Samples and Patient Data

Fresh HNSCC tumor specimens were obtained at the time of diagnosis. Adjacent normal tissue was sampled for comparison. This was taken from the same surgical field, as often done during the tumor survey, and did not put the patient at increased or additional risk. Standard biopsy techniques were performed, as were standard surgical resection when appropriate. Additional biopsies of normal tissue adjacent to the tumor mass were taken when required. Patients were excluded if their primary tumor was too small to allow both diagnostic biopsy and biopsy for the study. Patients were recruited and consented following an institutional review board-approved protocol before inclusion in the study.

Detailed clinical and demographic information was collected by physicians on all patients enrolled in the study and patients were prospectively followed-up to determine clinical outcome and disease progression. Patient information is entered on an ongoing basis into a secure clinical database system developed for the head and neck cancer program at the Albert Einstein College of Medicine. Patients undergo treatment for HNSCC, as deemed appropriate by the treating physicians blinded to miRNA results.

RNA Extraction

Fresh frozen tumor samples were prospectively collected from HNSCC patients at Montefiore Medical Center in the Bronx. Tumor tissue was snap-frozen in liquid nitrogen within 30 minutes of surgical resection or biopsy, and before treatment. Total RNA was extracted from an additional 50 to 100 mg of tissue using TRIzol by standardized protocol (Invitrogen, Carlsbad, CA). RNA was collected by alcohol precipitation and quantitated for microarray or real-time PCR analysis Selected samples were checked for integrity of RNAs and presence of microRNA peaks using an Agilent 2100 bioanalyzer (Agilent, Santa Clara, CA) and RNA pico chips as described by the manufacturer.

MicroRNA Microarrays

MiRNA oligonucleotide microarrays used in this study were custom printed at the Albert Einstein College of Medicine Microarray Facility. We printed a custom miRNA microarray that consisted of a set of antisense probes from Ambion Inc., Austin, TX (version 1) and the same oligonucleotide set described by Croce's group[19] that included sense strand probes, anti-sense controls, and selected miRNA precursor-specific probes representing a total 236 unique human miRNA genes. Each oligonucleotide was triple-spotted along with Ambion controls used for probe labeling and hybridization efficiency monitoring. MiRNAs were enriched from 5 μg of total RNA by fractionation using Ambion Flashpage polyacrylamide gels followed by column purification. Purified miRNAs were labeled by addition of polyA tails followed by chemical coupling of Alexa florescent dyes using the Vana miRNA labeling kit (Ambion) and hybridized using an Ambion hybridization kit recommended for these miRNA probes. Tumor sample RNA was labeled with Alexa 645 (red) and normal counterpart RNA with Alexa 555 (green). We also conducted dye flip experiments and yellow tests (comparison of identical RNA samples with both dyes on one microarray) to confirm the specificity of our microarray hybridizations.

Processing and Analysis of Microarray Gene Expression Data

Tumor versus normal signal intensities for each element on the array were calculated using the GenePix Pro 6.0 software package (Molecular Devices, Sunnyvale, CA). This software gives an integrated intensity per spot for each channel in addition to an integrated background count. In all subsequent analyses, we use the average background subtracted intensity for the two channels. For each spot, we calculated the mean intensity over the spot in the two channels and from this subtract the median of the background and $\log_2$ transformed. To correct for dye incorporation efficiency, fluorescence yield, and laser power used in scanning, we scaled the intensities from the two channels relative to each other, and computed an intensity-dependent normalization factor by first finding the rank invariant subset of the spots.[20] MiRNAs with missing signal intensity microarray expression data for two or more HNSCC cases were excluded from analysis. Furthermore, to account for labeling and hybridization efficiencies among samples, intensity ratios for each miRNA element were normalized by first taking the average value of the three spot replicates on the microarray and then correcting the values for each color channel using prespiked control oligonucleotides that are fluorescently labeled and hybridized along with the miRNA sample.[21]

Real-Time PCR

Both semiquantitative and quantitative real time PCR methods were used to validate and extend the microarray data. Initially, Ambion mirVana qRT-PCR primer sets were used along with TAQ polymerase as recommended by the supplier to validate microarrays. Each primer set (miR-21, miR-1, miR-133a, let-7d, miR-205, and miR-206 and 5S RNA) was individually titrated and a cycle chosen

such that the PCR product visualized by agarose gel analysis was in the exponential range of amplification. Tumor and normal samples were amplified and levels of products were estimated by gel electrophoresis and normalized to 5S RNA levels. Quantitative analysis of miRNA expression from large collections of tumor and normal samples was conducted by quantitative real-time PCR TaqMan protocol using a 7900 real time PCR apparatus (Applied Biosystems, Foster City, CA) exactly as recommended by the supplier. Results were expressed as a cycle threshold (C_T) value, which is inversely proportional to the sample starting copy number. Normalized (ΔC_T) values were computed by subtracting the C_T value (averaged across three replicates) of a small noncoding RNA control gene (*RNU 48*) from the raw C_T value of the miRNA element. To compare tumor and paired normal tissue, we generated the $\Delta\Delta C_T$ value by subtracting the ΔC_T value of the normal tissue from the ΔC_T value of the tumor. We then examined the $\Delta\Delta C_T$ distribution for each miRNA element graphically, assuming approximately equal amplification efficiencies between tumor and normal tissue.[21]

Statistical Analysis

We analyzed the association with cancer survival in a cohort of HNSCC patients undergoing treatment with curative intent in relation to miRNA level at diagnosis. Enrollment for the clinical study began in 2002 and patients were followed prospectively after histological confirmation of SCC. Time to event was measured from treatment start to the first instance of a local or regional recurrence, disease-free and overall survival, or to the last recorded follow-up visit date for censored patients. Survival and progression-free probability curves were estimated using Kaplan-Meier estimates. Cumulative incidence curves for loco-regional recurrence were also estimated, taking into account the competing risks of death and distant metastases. We investigated the potential effect of confounding by age, gender, ethnicity, smoking history, alcohol consumption, tumor location, disease stage, TNM status, treatment modality (primary resection +/− adjuvant therapy versus neoadjuvant therapy), and prevalence of human papillomavirus (HPV) (tested by MY09/11 PCR assay).[22,23] Adjusted models based on an *a priori* model containing known or suspected confounders were presented; however, we also examined all possible models using an exhaustive search of the model space. The *a priori* model included anatomical site (oropharynx, larynx, lip/oral), T stage (T0/T1 versus T2/T3), HPV status (HPV+ versus HPV−), and treatment (chemoradiation therapy yes/no). Hazard ratios (HRs) and corresponding confidence intervals for the adjusted models were estimated using Cox proportional hazards models. To test the null hypothesis that the regression coefficient was equal to zero, *P* values based on the Wald χ^2 test were computed and presented for each microRNA. Each event of interest (overall survival, loco-regional recurrence, distant metastases) was modeled separately, censoring at the occurrence of any competing event, to estimate the cause-specific hazard of event. To evaluate the overall impact of each covariate across all events of interest a single proportional hazards model, stratified by failure type, was estimated. This model allowed a different baseline hazard for each event type, but also allowed us to efficiently pool information across event type to generate an overall measure of variable importance in an adjusted model. Interactions between event type and covariate were examined for each covariate of interest. Robust standard errors using the sandwich estimator of Wei and colleagues[24] were computed to correct for any model misspecification.

Results

MicroRNA Profiling of HNSCC Tumors

We used custom-spotted oligonucleotide microarrays to make direct comparisons between HNSCC tumors and adjacent normal tissue to measure aberrant regulation of specific miRNA genes in HNSCC. We were able to generate a preliminary HNSCC miRNA signature of miRNAs over- and underexpressed in tumors relative to their matched normal counterparts (Table 1). The partial list shown includes 43 human miRNAs that were expressed, on average, twofold lower in tumors versus normal samples, and 6 miRNAs that were, on average, expressed by at least twofold higher in tumors versus normals. However, we observed some variability in miRNA expression across HNSCC patients. Assessing the frequency of HNSCC tumors over- or underexpressing miRNAs compared to their adjacent normal counterpart, we found only one miRNA with at least twofold higher expression in six of eight of patient tumors examined (represented by clones for miR-21, miR-021-prec, and mmu-miR-21_AS), whereas three human miRNAs were underexpressed in six of eight patients (miR-370, miR-199a-1-prec, miR-030b-prec).

Using a semiquantitative reverse transcription (RT)-PCR assay, we independently verified the microarray data in primary tumors and matching normal tissue for six different miRNAs from our preliminary HNSCC miRNA signature (*miR-21*, *miR-1*, *miR-133a*, *miR-205*, *miR-206*, and *let-7d*). Figure 1 shows some of the RT-PCR data comparing tumor (T) and matching normal (N) RNA from three HNSCC cases. In each case examined, we observed a general agreement between microarray and RT-PCR data. The concordance of microarray and RT-PCR data demonstrated that our microarray platform could accurately identify miRNAs that are dysregulated in HNSCC and therefore candidates for biomarker analysis of individual patient samples. Given the small initial sample size, we then set out to confirm the results on a larger sample set using quantitative real-time PCR and multivariable statistical analyses.

Table 1. List of the Highest Scoring MicroRNAs Corresponding to a Partial Expression Profile of HNSCC

MicroRNA	Mean fold change (tumor:normal)*
Underexpressed in tumors	
hsa-let-7f	0.29
hsa-miR-10b_AS	0.46
hsa-miR-124a_AS	0.34
hsa-miR-142-3p	0.43
hsa-miR-324-5p	0.23
hsa-miR-368	0.32
hsa-miR-370	0.26
hsa-miR-373*	0.44
hsa-miR-373*_AS	0.51
hsa-miR-422a_AS	0.43
hsa-miR-422b	0.16
hsa-miR-422b_AS	0.46
hsa-miR-424	0.27
hsa-miR-95_AS	0.46
hsa-miR-99a_AS	0.47
hsa-let-7a-2-prec-#1	0.54
hsa-let-7a-3-prec	0.29
hsa-let-7d-prec	0.40
hsa-miR-007-1-prec	0.30
hsa-miR-007-2-prec-#1	0.41
hsa-miR-009-1-#1	0.50
hsa-miR-009-3-#1	0.47
hsa-miR-016b-chr3	0.35
hsa-miR-030a-prec-#2	0.45
hsa-miR-030b-prec-#1	0.18
hsa-miR-030b-prec-#2	0.45
hsa-miR-093-prec-7.1 = 093-1	0.40
hsa-miR-106-prec	0.47
hsa-miR-125b-2-prec-#2	0.41
hsa-miR-128b-prec-#2	0.43
hsa-miR-130a-prec-#1	0.35
hsa-miR-135-1-prec	0.33
hsa-miR-140-#1	0.54
hsa-miR-155-prec	0.50
hsa-miR-192-2/3-#2	0.49
hsa-miR-199a-1-prec	0.24
hsa-miR-213-prec-#2	0.42
hsa-miR-216-prec-#1	0.43
hsa-miR-218-1-prec	0.36
hsa-miR-221-prec	0.24
hsa-miR-224-prec	0.20
miR1–2	0.44
miR133a-1	0.31
Overexpressed in tumors	
hsa-miR-021-prec-17-#2	4.44
hsa-miR-024-1-prec-#1	1.99
hsa-miR-151-prec	3.66
hsa-miR-199b-prec-#2	2.22
miR21	3.29
miR23b	1.54

*Tumor versus normal signal intensities for each element on the array were calculated using ratios of the mean spot intensities in the two channels over the eight HNSCC samples tested. AS, antisense probe; prec, precursor to the mature miRNA specific oligonucleotide probe.[19]

Quantification of MicroRNA Expression in HNSCC

We selected five miRNA genes from the list of miRNA above (miR-21, miR-1, miR-133a, miR-205, and let-7d) that might fit a HNSCC signature based on evidence in the literature and the preliminary results from the TvN microarray comparisons. For example, *miR-1* and *miR-133a* showed severely low expression levels relative to normal adjacent tissue (Table 1). We therefore hypothesized that these genes might discriminate between different HNSCC tumor behaviors. *MiR-205* was also selected because it has been shown to be highly enriched in HNSCC cell lines relative to other tumor types,[10] and therefore represents a cell-type-specific molecule that might regulate important downstream targets. Using primers for these candidate gene species, we then performed quantitative TaqMan real-time PCR analysis of an independent set of 104 HNSCC patient samples.

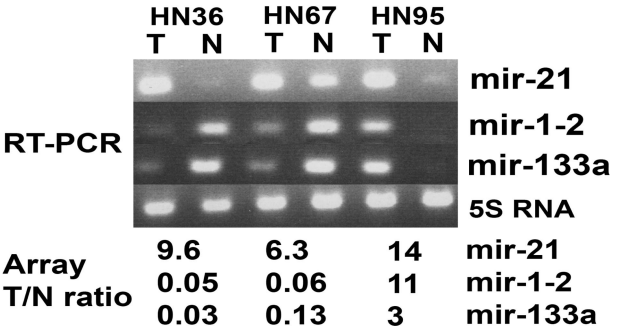

Figure 1. RT-PCR data comparing tumor (T) and matching normal (N) microRNA from three representative HNSCC cases. **Top:** Ratios of T/N expression derived from the microarrays are shown below the agarose gels displaying the PCR products derived from each RT-PCR reaction. 5S RNA was used as a control to show equal amounts of tumor and normal RNAs were included in each reaction. **Bottom:** Microarray data (array) shown as the ratio of tumor to normal expression (T:N ratio) for the samples used for RT-PCR is provided.

Patients were recruited from the Bronx, a high-risk area of New York City with a high incidence of head and neck cancer. Clinical characteristics of 104 patients with primary HNSCC tumors are shown in Table 2, and RNA prepared from these samples was used for our quantitative real-time PCR measurements. Matched tumor and normal adjacent tissue miRNA expression measurements were available on 95 of the 105 HNSCC patients tested. *RNU 48* RNA expression levels in all samples were used as a control for the amount of RNA used in the RT-PCR reactions. Measurements of *RNU 48* in tumor samples was consistent with a Gaussian distribution with a mean of 22.8 cycles, whereas normal samples were slightly skewed toward larger C_T values, and thus had a higher mean (C_T = 23.6). Measurements of the normalized *miR-205* RNA expression levels in tumors versus normal tissue from the same patients by real-time PCR revealed a significant difference and indicated that tumors averaged ~3.1-fold less *miR-205* than normal tissue ($\Delta\Delta C_T$ = +1.63, P value <0.001). In contrast, *miR-21* expression levels were higher in tumors versus normals by ~1.5-fold on average ($\Delta\Delta C_T$ = −0.58, P = 0.002) (Figure 2). Furthermore, looking at the distribution in miRNA expression across patients, two–fold lower *miR-205* levels were detected in 25% of tumors, whereas two-fold higher *miR-205* expression was seen in only 4.5% of cases. *Let-7d* showed a similar distribution, with 26% and 2% of cases showing lower and higher *let-7d* expression in tumor versus normals, respectively. In contrast, equal proportions (15%) of tumors showed two–fold lower or higher *miR-21* expression relative to their matched adjacent nor-

Table 2. Demographic and Clinical Characteristics of HNSCC Patients

Characteristic	*N*	%
Sex		
Male	71	68%
Female	33	32%
Age		
<60	41	40%
>60	63	61%
Ethnicity		
Hispanic	26	25%
Non-Hispanic	77	74%
Race		
White	65	62%
African-American/Black	30	29%
Other	2	2%
Anatomic site		
Oral cavity	31	30%
Oropharynx	32	31%
Hypopharynx	9	9%
Larynx	32	31%
Tumor Stage*		
I/II	24	23%
III/IV	80	77%
T status		
I/II	46	44%
III/IV	56	54%
N status		
I/II	40	40%
III/IV	56	54%
Treatment		
Primary chemo/RT	45	43%
Primary surgery	33	32%
Surgery -> chemo	22	21%
Smoking history		
Current	46	44%
Former	39	38%
Never	17	16%
Missing	2	2%
HPV status		
HPV16−	59	57%
HPV16+	37	36%
HPV status missing	8	8%
Total	104	100%

*Some cases pending histological confirmation. Totals may not add up to 104 because of missing data.

mal tissue. Interestingly, *miR-205*, *miR-21*, and *let-7d* demonstrated minor differences in expression levels with respect to tumor site within the head and neck, although these were within the limits of what may be considered biological variability (Table 3).

MicroRNA Expression and Clinical Outcome in HNSCC

Using the normalized real-time PCR results from 104 primary tumor samples, we also assessed the prognostic association with absolute levels of expression of each of the miRNA genes tested using prospective follow-up data collected on study patients. We observed a significantly shorter time to death (Figure 3) and loco-regional recurrence (Figure 4) in HNSCC patients showing combined lower absolute levels (higher ΔC_T) for *miR-205* and *let-7d*, indicative of increased risk with reduced expression in tumors. The prognostic impact of low *miR-205* and *let-7d* levels in tumors also appeared to be independent of anatomical site, tumor size (T-stage), treatment (chemo-radiotherapy), and HPV status (Table 4). The final model presented was based on *a priori* considerations, and takes into account many known or relevant predictors of progression and survival. Adjustment for anatomical site did not alter the parameter estimates substantively for any of the miRNAs, consistent with the univariate comparisons observed. More parsimonious models based on an exhaustive search across the model space did not significantly alter the results for *miR-205*, or *miR-205*, and *let-7d* combined (data not shown). To gain efficiency, we examined all relevant clinical outcomes simultaneously, estimating the overall effect of each variable stratified by event type. We did not observe any statistically significant interactions between event type and covariates, therefore the estimates presented are based on a summarized effect across all event types. We observed a significantly shorter time to cancer progression (loco-regional recurrence, distant metastasis) or death in HNSCC patients showing lower absolute levels (higher ΔC_T) for *miR-205* and *let-7d*, indicative of increased risk with reduced expression in tumors (Table 4). As illustrated by the unadjusted Kaplan-Meier and cumulative incidence curves, reduced expression of *let-7d* and *miR-205* combined remained a significant predictor of

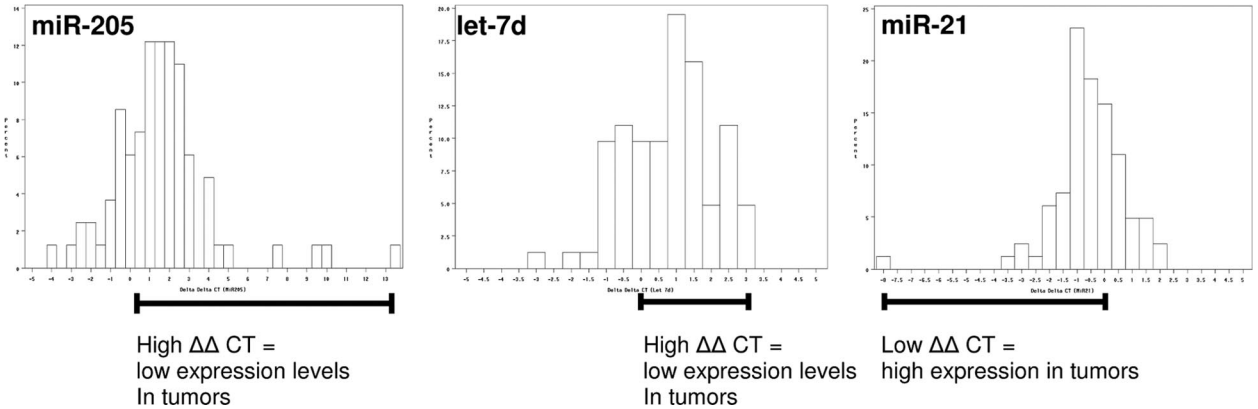

Figure 2. Distribution of tumor versus normal differences in normalized microRNA levels for *miR-205*, *miR-21*, and *let-7d*. Matched tumor and normal fold differences in microRNA TaqMan $\Delta\Delta C_T$ levels normalized to the *RNU48* control gene shown.

Table 3. Difference in Tumor MicroRNA Levels by Anatomic Site

Anatomic site	miR-205 (n = 104)	Effect (SE)*	miR-21 (n = 104)	Effect (SE)*	let-7d (n = 104)	Effect (SE)*	miR-133 (n = 41)	Effect (SE)*	miR-1 (n = 41)	Effect (SE)*
Oropharynx	−0.86	1.0 (ref)	−2.11	1.0 (ref)	3.72	1.0 (ref)	7.56	1.0 (ref)	6.70	1.0 (ref)
Larynx/hypopharynx	−0.53	0.33 (0.54)	−2.27	−0.15 (0.26)	3.73	0.003 (0.23)	6.64	−0.92 (3.97)	6.33	−0.37 (3.78)
Lip/oral cavity	−0.08	0.78 (0.58)	−2.74	−0.62 (0.28)	3.80	0.08 (0.24)	5.00	−2.57 (1.38)	4.67	−2.04 (1.31)

*Effect estimates and standard errors (SEs) for relative differences in microRNA expression are shown by β coefficient based on a linear regression model showing the difference in normalized ΔC_T tumor levels using oropharynx as the reference, adjusting for tumor site.

cancer progression (HR = 4.61, P value <0.0001), independent of anatomical site, T-stage, treatment, or HPV.

Discussion

One of the most promising aspects of molecular classification is the possibility of isolating smaller subsets of genes whose expression (or lack thereof) correlates significantly with clinical parameters, and the ability to construct a disease-specific assay that can be used to classify cancers and predict clinical outcome.[25] In one of the early studies to link gene expression profiling to treatment response, Hanna and colleagues[16] identified 60 tumor-related genes from a cDNA microarray containing 1187 genes that could successfully predict the radiation response of tumor samples. In oral cancer, Warner and colleagues[17] identified 23 differentially expressed genes that correlated with tumor stage (III to IV) and metastasis. Gene expression signatures have also been identified that are associated with recurrence of HNSCC disease.[17] This study represents the initial use of miRNAs to classify and predict behavior of HNSCC.

Most of the existing miRNA data for HNSCC comes from work with cell lines in culture. A real-time PCR study of 222 miRNAs in 32 cancer cell lines that included 4 head and neck lines confirmed that miRNA expression patterns in HNSCC cells could be distinguished from the cell-type specificity of the other 28 lines that included lung, pancreas, prostate, breast, colorectal, and blood cell types.[9] This result is consistent with the prevailing

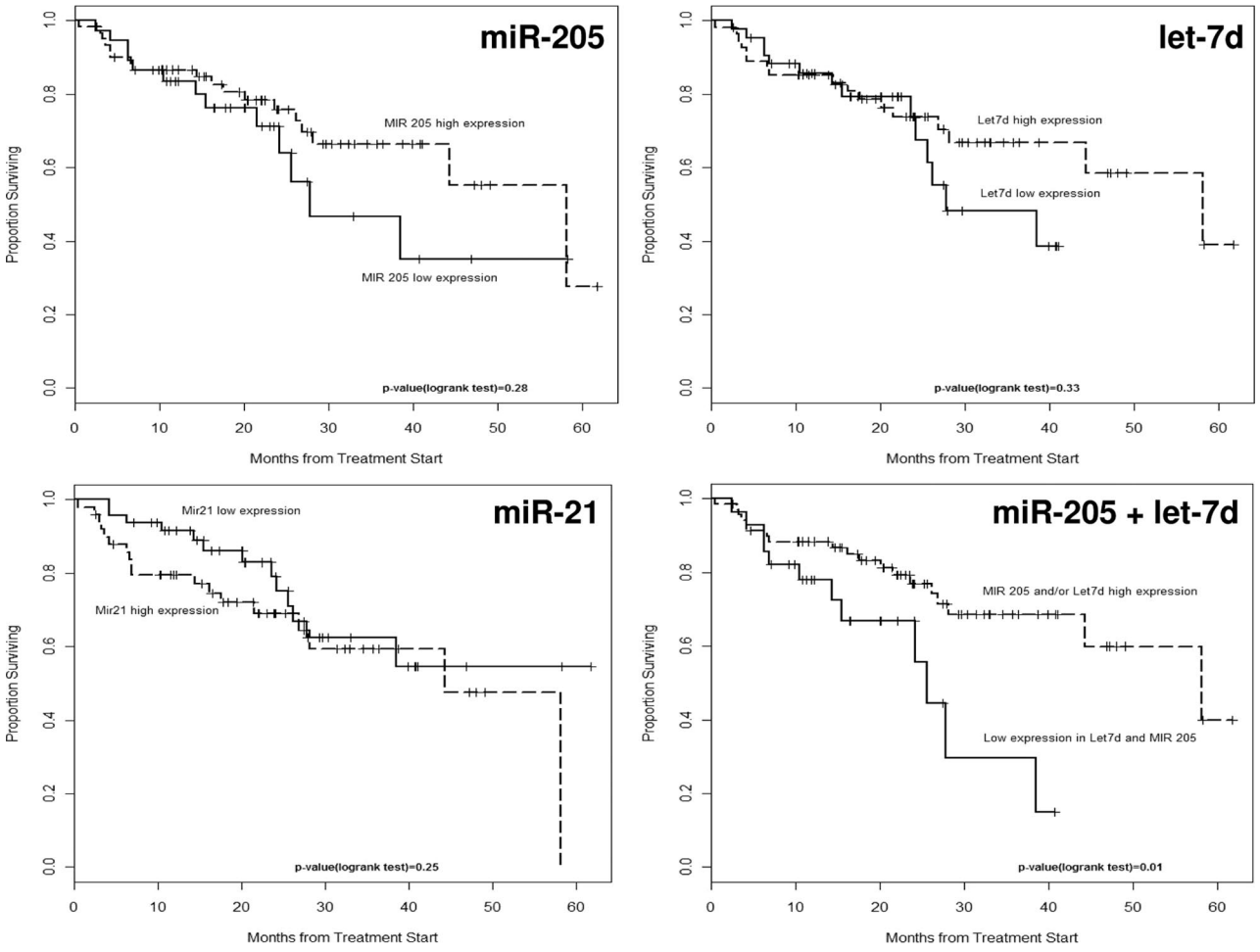

Figure 3. Kaplan-Meier plots for overall survival by *miR-205*, *miR-21*, and *let-7d* expression individually and combined.

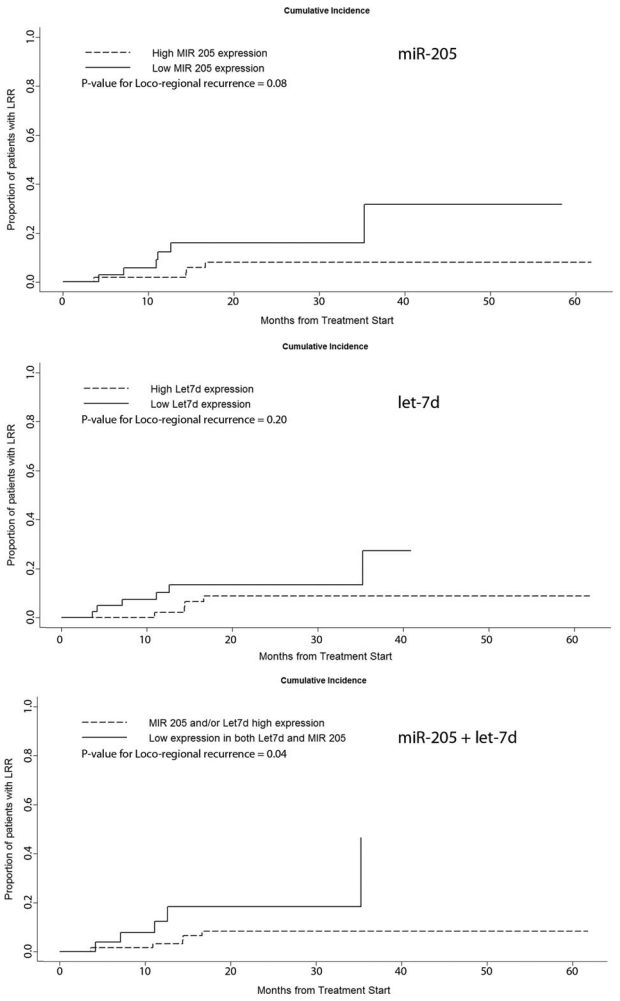

Figure 4. Cumulative-incidence plots for loco-regional recurrence by *miR-205* and *let-7d* expression individually and combined. Cumulative incidence curves for loco-regional recurrence were also estimated, taking into account the competing risks of death and distant metastases. *P* values for the comparison of cumulative incidence curves were computed based on the approach of Gray.[41]

idea that miRNAs are differentially expressed in tissues and during altered growth.[26,27] In addition, *miR-205* is a relatively enriched species in head and neck, having higher expression in the HNSCC lines than any of the other cell lines derived from different cancers.[24] Comparison of miRNA expression profiles in nine HNSCC cell lines revealed 33 highly expressed and 22 lowly expressed miRNAs.[10] Among the highly expressed miRNAs were *miR-212* and *miR-205*. These data comparing cell lines from different tumor origins revealed that *miR-205* is abundant in its expression in squamous cells in head and neck tissues (relative to other cell types in other tissues) but they obviously could not address the issue of *miR-205* expression in tumors compared with normal tissue or of HNSCC tumors compared among themselves for correlation with clinical data. Our data are novel in that we observed that it is the repression of this cell-type-enriched miRNA in patient tumors that is important as a prognostic predictor independent of anatomical site, treatment, or disease stage at presentation.

MiR-205 therefore, seems to act as a tumor suppressor in HNSCC, and reduced expression of this *miRNA* should result in increased expression of target oncogenes. To demonstrate this, we attempted to identify potential *miR-205* targets by looking for mRNAs whose expression levels that were inversely correlated with *miR-205* levels in the same HNSCC tumors. We then used TargetRank to predict which among the highly correlated genes were also theoretical miRNA targets, representing potential direct *miR-205* targets.[28] One gene that met these two selection criteria was dihydrofolate reductase (*DHFR*). In 16 ($N = 16$) HNSCCs in which primary tumors and adjacent normal tissue measurements by both cDNA microarray and TaqMan RT-PCR data were available for comparison, we observed a high correlation ($corr = 0.89$) between *miR-205* (low expression) and *DHFR* (high expression). TargetScan predicts a score of 0.422 (on a scale of 0 to 1) with four conserved seed matches in the *DHFR* gene. *DHFR* plays a critical role in folate metabolism, and represents the target of methotrexate, a drug commonly used in preventing tumor growth. *DHFR* activity is also associated with the *p53* tumor suppressor protein because both are targeted for degradation through the *MDM2* ubiquitin ligase pathway. In addition, it was recently shown that a specific *MDM2* polymor-

Table 4. Hazard Ratios (HRs) for Each MicroRNA Expression in Tumors with Survival and Loco-Regional Recurrence

MicroRNA	Univariate HR*	*P* value	Adjusted HR†	*P* value
Overall survival				
miR-205 (low versus high)	1.49	0.28	2.51	0.025
miR-21 (high versus low)	0.67	0.25	1.00	0.995
miR-let7d (low versus high)	1.44	0.33	1.73	0.166
Loco-regional recurrence‡				
miR-205 (low versus high)	3.19	0.07	4.19	0.049
miR-21 (high versus low)	1.46	0.55	1.94	0.281
miR-let7d (low versus high)	2.42	0.17	2.34	0.132
Survival or disease progression§				
miR-205 (low versus high)	NA		2.93	0.008
miR-21 (high versus low)	NA		1.81	0.658
miR-let7d (low versus high)	NA		2.30	0.017

*Categorized into a binary variable using a mean ΔC_T cutoff.
†Adjusted for *t*-stage, anatomic site, treatment, and HPV.
‡Restricting to patients with no evidence of disease after treatment.
§Local, regional recurrence, distant metastases, or death. Univariate models not estimated.

phism, SNP309, is correlated with early tumor onset in HNSCC and poor prognosis in other tumors.[29] These pathways are additionally linked through *p14^{ARF}* which is frequently lost or mutated in HNSCC. The activity of *p14^{ARF}* results in increased degradation of *DHFR* and resistance to folate antagonists in cells with nonfunctional *p53*.[30] Our results suggest that these pathways may intersect and that *miR-205* may be involved in the regulation of these events that determine these cancer-related phenotypes. Further dissection of these pathways and direct experimental determination of miR-205 target genes in HNSCC cell lines and confirmation of these targets in specific head and neck clinical samples should help to understand mechanisms involved in these responses.

In our study, we find repression of miRNAs to be more common than overexpression in human tumors compared to normal tissue. This is consistent with other studies that looked at human oral specimens, and with the hypothesis that a subset of miRNA genes may be epigenetically silenced by hypermethylation of CpG island sequences within promoter or exon regions.[31] Alternative mechanisms proposed for altered levels of miRNA genes in cancer include alterations in copy number, chromatin modifications, and regulation by transcription factors. One example includes the *let-7* family of genes. It has been recently reported that the *c-myc* oncogene contributes to widespread repression of at least 21 distinct miRNA transcription units including *let-7* family members and *let-7d* specifically.[32] The *let-7* gene family consists of at least nine genes within eight transcription units. *C-myc* is frequently overexpressed in HNSCC tumors and it is possible that one important consequence of this for the oncogenic effects of *myc* in HNSCC is the repression of *let-7* miRNA. Low levels of expression of various members of this multigene family in tumors relative to normal tissue have been shown to predict poor prognosis in lung cancer.[5,6] Our results add HNSCC as another cancer type in which pathways regulated by *let-7* are critical for tumor behavior.

It will also be important to identify and experimentally confirm downstream targets of *let-7d* because these genes could prove to become new potential therapeutic targets in the future. One experimentally verified target of *let-7* miRNAs is the *KRAS* oncogene.[33] *HMGA2* is another *let-7* target gene and this gene is also regulated in part in hypoxic conditions by *miR-98* in the HNSCC cell lines SCC-4 and SCC-9.[34] *HMGA2* activity is associated with enhanced sensitivity to the topoisomerase II doxorubicin and high levels in lung tumors is correlated with poor prognosis and metastasis.[35] We also looked for mRNAs whose expression levels were inversely correlated with *let-7d* levels in the same HNSCC tumors in an attempt to identify additional target genes of possible interest. Many genes show highly significant inverse correlations with *let-7d* expression, although many of the identified target genes represented unidentified ESTs, and no known genes contained seed regions that matched *let-7d* binding sites. Further studies are needed to identify target *let-7d* target genes in HNSCC. Again, experimental determination of *let-7* targets in HNSCC cells will be necessary to make clearer the mechanisms responsible for the critical role of *let-7d* in HNSCC tumor phenotypes.

Expression levels of *miR-21* RNA were not correlated with any of the clinical parameters that we measured, however, this miRNA has experimentally verified target genes that have been shown to be altered in tumors, one of which is *PDCD4*, a tumor suppressor protein involved in invasion and metastasis.[36,37] We have derived extensive cDNA microarray data comparing primary HNSCC tumors to their normal counterparts in the same patient (data not shown). Examination of these tumor/normal datasets revealed that *PDCD4* mRNA is down-regulated twofold or more in 15 of 26 cases examined. Thus, we imply that this *miR-21* target gene identified during manipulation of tissue culture lines is also relevant to actual patient samples. Additional *miR-21* targets identified along with *PDCD4* include the actin protein *ACTA2*, and the anti-proliferative protein encoded by the *BTG2* gene.[37] Examination of our cDNA microarray data again showed that *ACTA2* expression was twofold lower or less in 7 of 26 HNSCC cases, whereas *BTG-2* was lower in 9 of 26. These correlations between miRNA expression levels and potential mRNA targets in clinical samples would seem to support the relevance of at least some targets identified in tissue culture models.

Another experimentally confirmed *miR-21* target gene is the *PTEN* tumor suppressor.[38] *PTEN* tumor suppressor is frequently altered in HNSCC[39,40] and loss of heterozygosity caused by homozygous deletion or allelic loss and point mutation have been documented. However, in HNSCC neither of these mechanisms could account for a majority of the tumors with reduced *PTEN* protein expression.[40] *PTEN*, however, is a confirmed target of *miR-21*, the only miRNA overexpressed in all solid tumors examined as well as some additional cancers.[3] *PTEN* encodes a phosphatase that can inhibit growth and/or survival pathways including AKT/PI3K, and its function is altered in advanced tumors of various types, including breast, lung, gastric, and prostate. As in HNSCC, the frequency of loss of heterozygosity at the *PTEN* locus in these tumors is relatively small (less than 10%). Using tissue microarrays to measure *PTEN* protein levels in ($n = 46$) primary HNSCC tumors used for miRNA measurements, we observed that *miR-21* was up-regulated in tumors where *PTEN* expression was absent, albeit not significantly ($P = 0.10$). Looking at differences in *miR-21* RNA levels in tumor versus normal tissue by case, the majority of HNSCC (79%) that showed no evidence of *PTEN* expression had higher *miR-21* tumor levels, whereas in cases where *PTEN* was present, only 46% of HNSCC showed an absolute increase in *miR-21* levels ($P = 0.03$), and no cases showed a greater than twofold difference in tumor versus adjacent normal. We did not observe these same correlations with *PTEN* mRNA as measured by microarray analysis. These observations would suggest that translational inhibition of *PTEN* mRNA via *miR-21* is an alternative mechanism to loss of heterozygosity and mutation for the silencing of the *PTEN* tumor suppressor. This miRNA may therefore play a role in HNSCC risk or tumor development, although we do not find any association with cancer progression in the head and neck.

In conclusion, microRNAs *miR-205* and *let-7* expression levels can be used as prognostic markers of head and neck cancer survival and recurrence. These observations may have diagnostic and therapeutic implications for the management and treatment of HNSCC patients. Our results also expand the known pathways and genes that are important for the behavior of this disease and represent an important source of new avenues of research in head and neck cancer.

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